REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
29-12-2008	Final Report	02/01/2007 - 08/15/07

4. TITLE AND SUBTITLE

Structural, thermodynamic, and functional mechanisms of adaptations WrbA and AdoMetDC proteins in extremophilic organisms

5a. CONTRACT NUMBER

5b. GRANT NUMBER
FA9550-07-1-0159

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

George Makhatadze

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Pennsylvania State University
College of Medicine/M.S Hershey
Medical Center
P.O. Box 850, Hershey, PA 17033-0850

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Air Force Office of Scientific Research /NL
875 North Randolph Street, 4036
Arlington, VA 22203

10. SPONSOR/MONITOR'S ACRONYM(S)
AFOSR

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Unlimited release

AFRL-SR-AR-TR-08-0157

13. SUPPLEMENTARY NOTES

20080331085

14. ABSTRACT

One of the open questions of structural biology is the understanding of the mechanisms by which enzymes adapt to extreme temperatures, both high (thermophilic) and low (psychrophilic). A model protein used in this study, S-adenosyl-methionine decarboxylase (AdoMetDC), is a key enzyme in the polyamine biosynthesis and thus its activity should be strongly dependent on the environmental variables such as temperature. To date we completed the experimental characterization of the thermophilic AdoMtDC from *Trematoga maritima*. The processing of TmAdoMetDC that leads to catalytically active enzyme is undetectable at room temperature, but increases with the increase in temperature ($k=0.41\pm0.08$ h^{-1} at 65°C). The binding constant for MMTA, an inhibitor that mimics natural substrate S-adenosyl-methionine, was found to be on the order of 1 μM. A paper that summarizes these results is currently in preparation. The genes for AdoMtDC from five different bacteria have been cloned: *Leptospira interrogans* (psychrophile), *Exiguobacterium sibiricum* (psychrophile), *Petrotoga mobilis* (thermophilic anaerobe), and *Oceanobacillus iheyensis* (halophilic). All these proteins have been expressed and purified, and structural (crystallization for x-ray analysis), biophysical (e.g. stability, oligomerization) and biochemical (activity profile as a function of temperature and salt concentrations, activation energy) experiments are currently in progress.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			George Makhatadze
none	none	none		20	19b. TELEPHONE NUMBER *(include area code)* (518) 276-4417

1. Cover Sheet

Principal Investigator Name: George Makhatadze

Institution Name: Pennsylvania State University

Institution Address: College of Medicine

M.S Hershey Medical Center

P.O. Box 850

Hershey, PA 17033-0850

Agreement Number: FA9550-07-1-0159

2. Objectives

No changes

3. Status of Efforts

One of the open questions of structural biology is the understanding of the mechanisms by which enzymes adapt to extreme temperatures, both high (thermophilic) and low (psychrophilic). A model protein used in this study, S-adenosyl-methionine decarboxylase (AdoMetDC), is a key enzyme in the polyamine biosynthesis and thus its activity should be strongly dependent on the environmental variables such as temperature. To date we completed the experimental characterization of the thermophilic AdoMtDC from *Termatoga maritima*. The processing of TmAdoMetDC that leads to catalytically active enzyme is undetectable at room temperature, but increases with the increase in temperature ($k=0.41\pm0.08$ h^{-1} at 65°C). The binding constant for MMTA, an inhibitor that mimics natural substrate S-adenosyl-methionine, was found to be on the order of 1 μM. A paper that summarizes these results is currently in preparation. The genes for AdoMtDC from five different bacteria have been cloned: *Leptospira interrogans* (psychrophile), *Exiguobacterium sibiricum* (psychrophile), *Petrotoga mobilis* (thermophilic anaerobe), and *Oceanobacillus iheyensis* (halophilic). All these proteins have been expressed and purified, and structural (crystallization for x-ray analysis), biophysical (e.g. stability, oligomerization) and biochemical (activity profile as a function of temperature and salt concentrations, activation energy) experiments are currently in progress.

4. Accomplishments/New Findings

During the short duration of this project (start February 2007 end August 2007) we spent the effort on the Specific Aim 1, and in particular on understanding the structural and thermodynamic mechanisms in protein adaptation to extreme thermal conditions using AdoMetDC proteins from psychrophilic, mesophilic and thermophilic organisms.

One of the open questions of structural biology is the understanding of the mechanisms by which enzymes adapt to extreme temperatures, both high (thermophilic) and low (psychrophilic). To this end we initiated experiments that address this question using a family of bacterial S-adenosyl-methionine decarboxylase (AdoMetDC). AdoMetDC belongs to a class of pyruvoyl-dependent amino acid decarboxylases that catalyze the removal of the carboxylate group from S-adenosylmethionine (S-AdoMet) to form decarboxylated DC-S-AdoMet. This acts as the n-propylamine donor for spermine and spermidine synthases to synthesize spermidine and spermine from putrescine. Since AdoMetDC is a key enzyme in the polyamine biosynthesis, its activity should be strongly dependent on the environmental variables such as temperature. This makes this protein an excellent model to study the mechanisms of adaptation to extreme thermal environment.

The genes for AdoMtDC from five different bacteria have been cloned: *Bacilus subtilis* (mesophile), *Leptospira interrogans* (psychrophile), *Exiguobacterium sibiricum* (psychrophile), *Petrotoga mobilis* (thermophilic

anaerobe), and *Oceanobacillus iheyensis* (halophilic), in addition to the protein from *Thermatoga maritima* (thermophilic archea) (see Figure 1 for sequences). All of the proteins have been expressed and purified, and structural (crystallization for x-ray analysis), biophysical (e.g. stability, oligomerization) and biochemical (activity profile as a function of temperature and salt concentrations, activation energy) experiments are currently in progress. Below, we report preliminary results obtained during this funding term.

Protein Cloning Expression and Purification

All proteins were expressed with the N-terminal tag that included 6xHis tag as well as TEV protease cleavage site (MHHHHHHENLYFQG). TEV protease cleaves the peptide bond preceding G. Thus all proteins have an additional N-terminal residue. The advantage of the 6HisTEV construct is that it allows rapid protein purification (using Ni-NTA-sepharose) and the tag can be easily removed after purification. The first step was to separate His-tagged protein from the rest of cellular debris and proteins. The second Ni-NTA step was after TEV protease cleavage to separate untagged from the 6HisTEV tagged protein. In addition, proteins were passed twice through gel filtration column (Sephadex G100, 2.5x150 cm) after each of the two steps of Ni-NTA steps. The purity of the final protein prep was checked by SDS-PAGE and MALDI-TOF.

Structural integrity of the purified proteins was checked using circular dichroism (CD) spectroscopy. All proteins showed spectra typical for folded proteins (Figure 2). The overall intensity of the CD spectrum for PETMO is

somewhat lower than for the other proteins, but this is probably related to the accuracy of the extinction coefficient determination and will be addressed in our future experiments.

Oligomerization state of the protein is solution was analyzed using analytical ultracentrifugation (AUC). Typical AUC profile is shown in Figure 3. The results of analysis are given in Tables 1 and 2. At neutral pH, the AUC results suggest that all studied proteins behave as a single species with the molecular mass corresponding to the protein dimer. The only difference is LEPIN, which sediments with larger apparent molecular mass. The data can be fit to a model in which the protein oligomerization state is an equilibrium between dimers and tetramers, with the apparent dimer-tetramer dissociation constant of $(4\pm2)\cdot10^5$ M^{-1}.

Detailed studies of the effect of pH on the structure and oligomerization state in solution has been done so far for the TmAdoMetDC protein. Figure 4 shows the CD spectra of TmAdoMetDC at different pH. It appears that the extremely low (pH 2.06) or high pH (pH 8.5) lead to a decrease in the ellipticity suggesting some conformational changes. In the pH range from 3.3 to 7.5 the spectra are overlapping suggesting that there are no pH-induced changes in the protein conformation. Table 2 shows the effect of pH on the oligomerization state of TmAdoMetDC in solution. At neutral pH, the protein is obligatory dimer. However, decrease in pH lead to increase in population of monomers. The dissociation constant at low pH is on the order of $(7\pm1)\cdot10^3$ M^{-1}. Overall, the changes in K_{dis} represent a titration curve, suggesting that ionization of protein groups might be responsible for this effect (Figure 5).

To test this hypothesis we calculated the pKa of TmAdoMetDC based on the crystal structure of the dimer and monomer. Since there are no changes in the CD spectra for the most of pH values, it was assumed that the structure of monomer is identical to that of monomer when part of the dimer. Calculations were done using MCCE. Multi-conformation continuum electrostatic (MCCE) is a hybrid of continuum electrostatics and molecular mechanics. MCCE starts with atomic coordinates from the Protein Data Bank (PDB) as inputs and solves the Poisson-Boltzmann equations by the finite difference method for multiple conformers. MCCE keeps the protein backbone rigid while letting side-chains of polar and ionizable groups sample additional rotamer positions. Thus, residues have pre-assigned conformers differing in atomic positions or ionization state. Using the calculated pKa values we constructed the pH profile of net charge of the monomer and dimer of the TmAdoMetDC. The difference between the two titration profiles was compared to the experimentally measured dissociation constant. The results are shown in Figure 5, and support the notion that the ionization of the residues on the dimer interface of TmAdoMetDC might be responsible for the pH induced dissociation of the TmAdoMetDC dimer.

Functional Assay

Functionality assays were so far performed with the TmAdoMetDC protein. Activation of AdoMetDC enzymes occurs via a self-processing reaction. The overall mechanism for the TmAdoMetDC self-processing reaction is the same as in the other pyruvoyl-dependent enzymes. It has been postulated that an ester

intermediate is formed when the side chain oxygen of S63 performs a nucleophilic attack on the main chain carbonyl carbon atom of E62. A beta-elimination occurs across the Cα and Cβ bond of Ser^{63}, resulting in a dehydroalanine at residue 63 and a new C terminus at residue 62.

The processing of TmAdoMetDC that leads to catalytically active enzyme is strongly temperature dependent. Processing is extremely slow (undetectable) at room temperature, but increases with the increase in temperature. Analysis of the data shows that at 65°C the first order rate constant for processing is 0.41±0.08 h^{-1} (Figure 6). The processed TmAdoMetDC does have ability to bind the substrate analog MMTA (5'-Dexoy-5'-dimethyl-thioadenosine), that mimics natural substrate AdoMet. This was established using isothermal titration calorimetry (ITC). Figure 7 compares the ITC titration profiles for TmAdoMetDC-WT, TmAdoMetDC-WT* (obtained after exposing protein to 65°C for 24 hours), and TmAdoMetDC-S63A (active site mutant). It is clear that only the TmAdoMetDC-WT* can bind the substrate MMTA. By performing ITC experiments at several different temperatures, we obtained the complete thermodynamics of MMTA binding to TmAdoMetDC-WT* (Figure 8). Two features are important: 1. There is a strong enthalpy-entropy compensation, with enthalpy always favoring the binding. 2. The heat capacity changes upon binding that define the temperature dependence of the enthalpy and entropy of binding is negative. Both these observations are consistent with the hydrophobicaly driven association process.

AdoMetDC proteins from plant (potato) and mammals (human) are activated by binding of putrescine. We thus tested the ability of TmAdoMetDC-WT* to bind this polyamine. The binding assays were done using ITC and also by fluorecescence spectroscopy. Both these techniques did not show any putrescine binding to TmAdoMetDC-WT*. The lack of binding was independent of whether TmAdoMetDC-WT* was presaturated with MMTA or not. This suggests that bacterial AdoMetDC enzymes do not require putrescine for activation and thus are not regulated by this compound.

Table 1. Results of the analytical ultracentrifugation experiments

Protein Loading OD	Final estimate (20000 rpm)	Final estimate (25000 rpm)	Final estimate (30000 rpm)	Final estimate (Global fit)	Final estimate (Global fit)	Theoretical Molecular Mass (monomer)	Theoretical Molecular Mass (dimer)
EXIS 0.1 ou	27996	27996	28004	27996	*27289*	*14087*	*28174*
EXIS 0.3 ou	31148	28681	26125	27291			
PETMO 0.1 ou	25881	28267	26154	26117	*28419*	*14925*	*29850*
PETMO 0.3 ou	30672	28390	30480	28924			
LEPIN 0.1 ou	55832	53794	46517	53752	*49897**	*14262*	*28524*
LEPIN 0.3 ou	53721	49019	46836	49567			
OCENI 0.1 ou	37295	29364	29543	29576	*28005*	*13936*	*27872*
OCENI 0.3 ou	30552	27996	26611	27515			

Buffer: 10 mM NaH_2PO_4, 1 mM DTT, and 0.5 mM EDTA, 100mM NaCl, pH=7.5

* Fit to a dimer-tetramer model yealds to a dissociation constant $(4\pm2)\ 10^5\ M^{-1}$.

Table 2. Results of the analytical ultracentrifugation experiments for TmAdoMetDC at different pH

pH	Model	Molecular Mass	$K_{mon\text{-}dim}$
2.06	Monomer<-> Dimer		$7.7 \cdot 10^3$
2.5	Monomer<-> Dimer		$6.4 \cdot 10^3$
3.3	Monomer<-> Dimer		$2.4 \cdot 10^5$
4.5	Monomer<-> Dimer		$1.6 \cdot 10^5$
5.5	Monomer<-> Dimer		$2.3 \cdot 10^6$
6.5	Obligatory Dimer	33.8±0.2 kDa	$>10^8$
7.5	Obligatory Dimer	33.8±0.1 kDa	$>10^8$
8.5	Monomer<-> Dimer		$3.4 \cdot 10^5$
9.5	Monomer<-> Dimer		$1.3 \cdot 10^5$

Figures

Figure 1. Sequence alignment of AdoMetDC proteins from different bacterial sources: *Leptospira interrogans* (LEPIN or Li), *Exiguobacterium sibiricum* (EXISI or Es), *Bacilus subtilis* (BACSU or Bs), *Oceanobacillus iheyensis* (OCEIH or Oi), *Petrotoga mobilis* (PETMO or Pm), *Thermatoga maritima* (THEMO or Tm) Numbering is that of THEMO. Secondary structure as determined from the 3D-structure of TmAdometDC (1TLU) is given in the last row (S – sheet; H – helix).

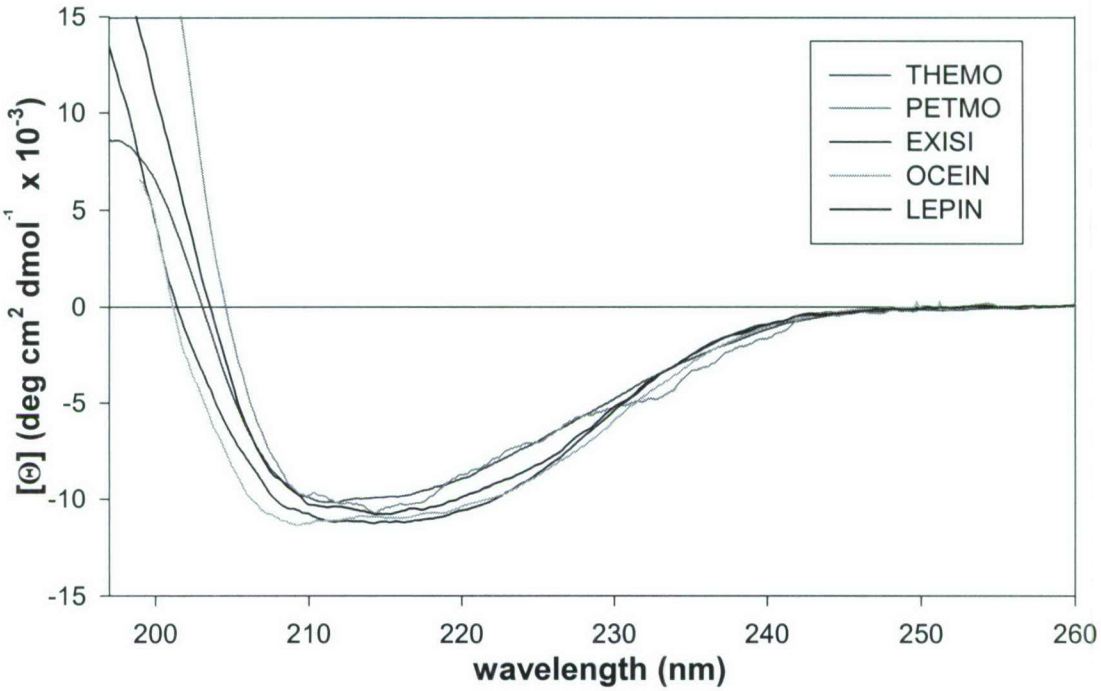

Figure 2. Comparison of the far-UV CD spectra for AdoMetDC proteins.

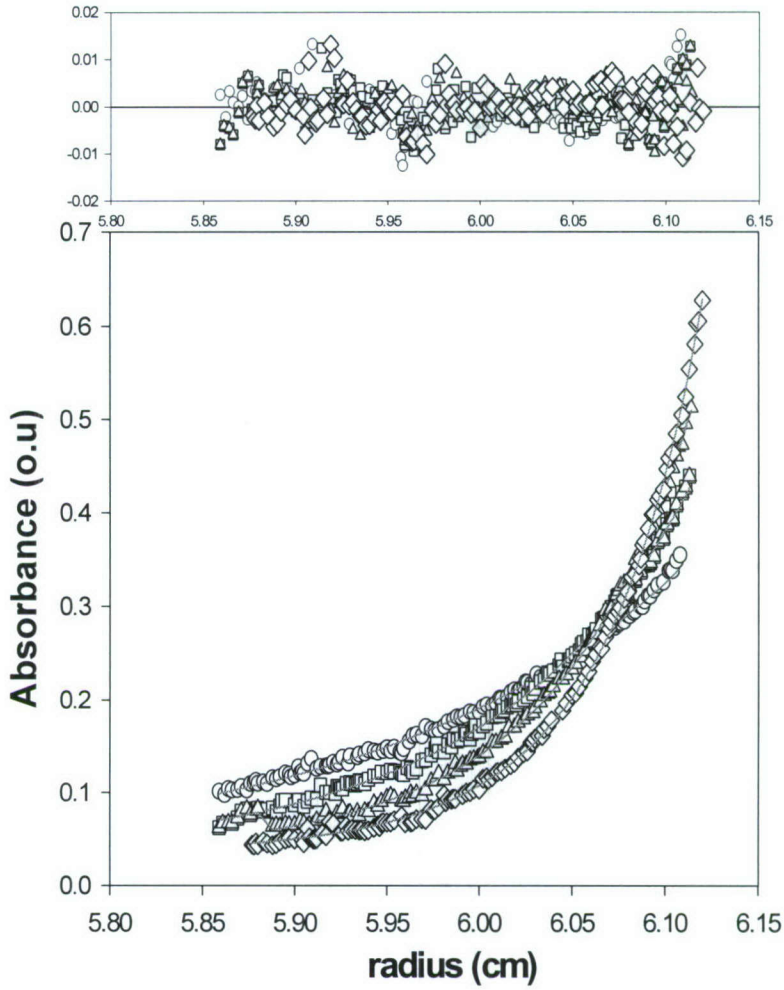

Figure 3. Analytical centrifugation profiles of TmAdoMetDC at pH 2.06 at 4°C collected at the rotor speeds of 20,000, 25,000 and 30,000 rpm. Symbols show the experimental data points solid lines fits to the monomer-dimer equilibrium.

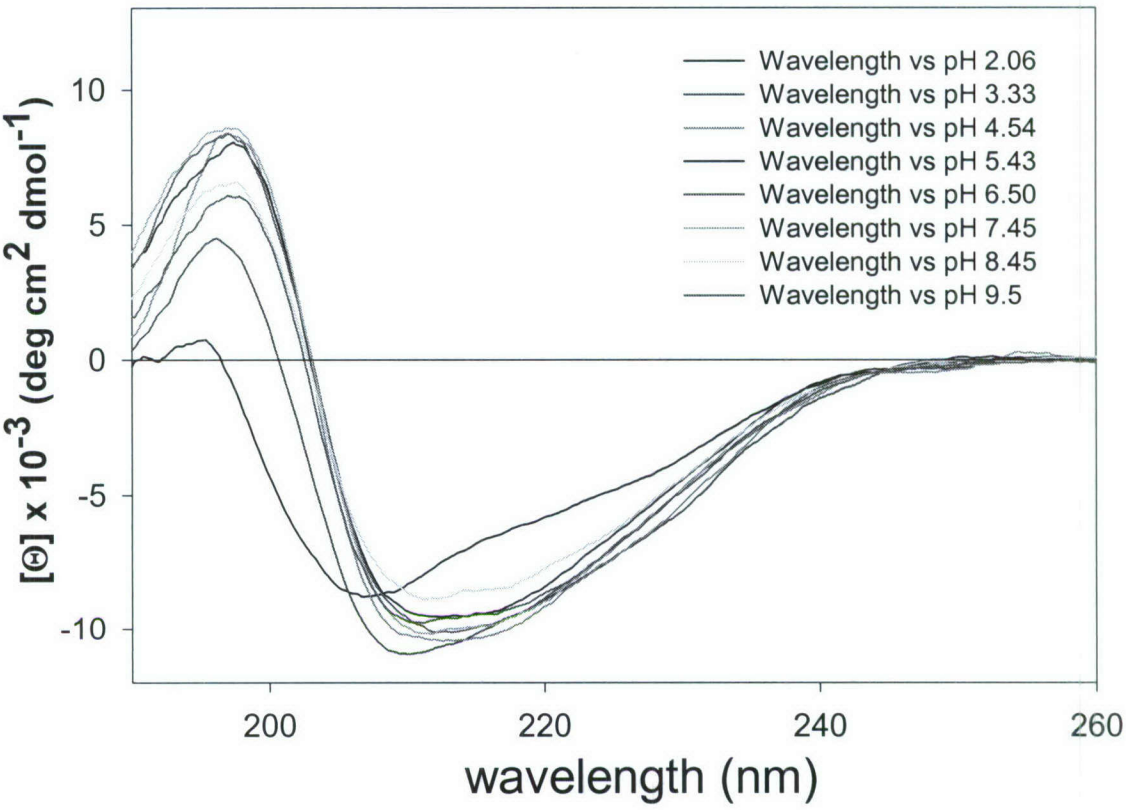

Figure 4. Effect of pH on the far-UV CD spectrum of TmAdoMetDC.

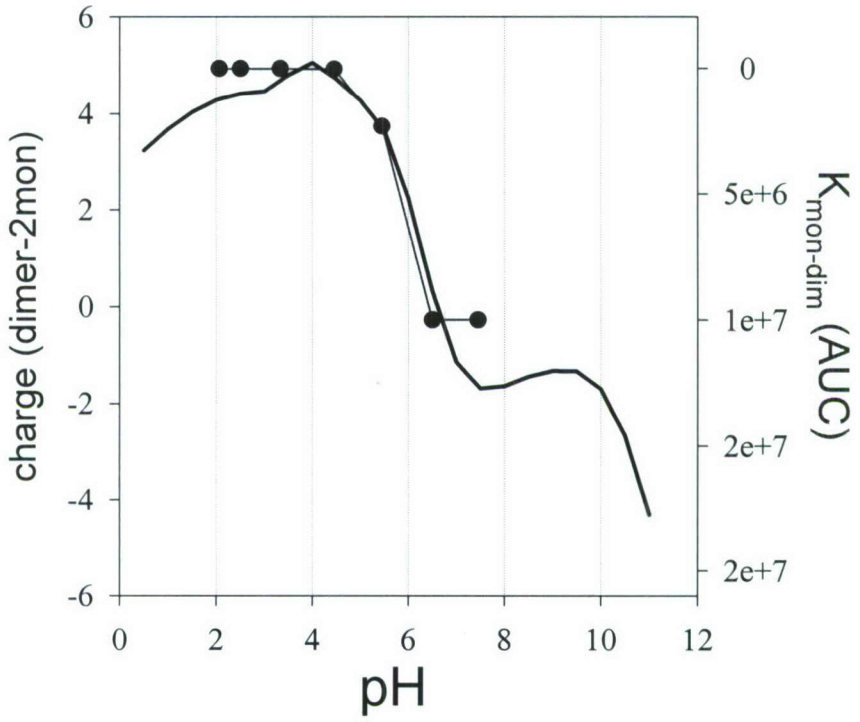

Figure 5. Dependence of the monomer-dimer dissociation constant for TmAdoMetDC as a function of pH. Symbols – experimental data obtained from the AUC experiments, solid lines – difference in the net charge of monomer and dimer as calculated using MCCE.

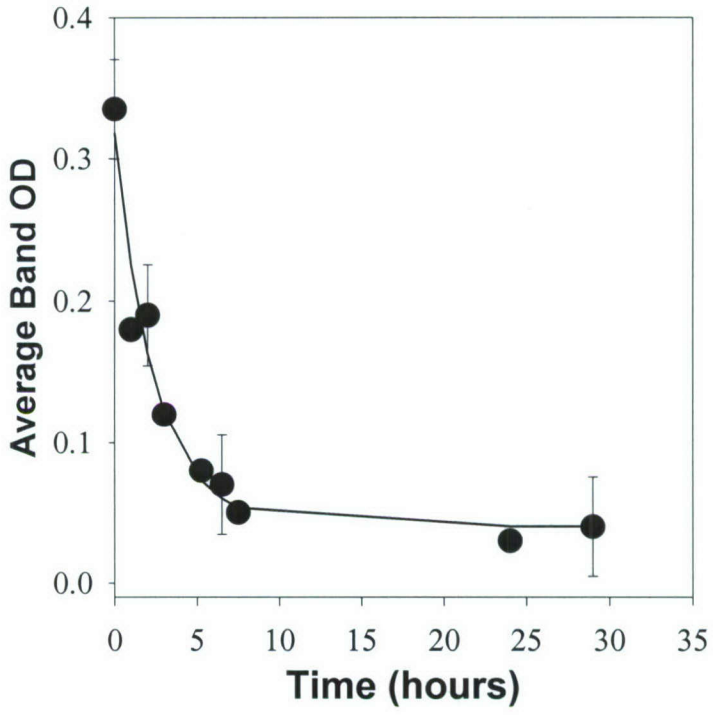

Figure 6. Time dependence of the autoprocessing for TmAdoMetDC at 65°C. Symbols – experimental data obtained from the SDS-PAGE, solid line – fit to the first order rate.

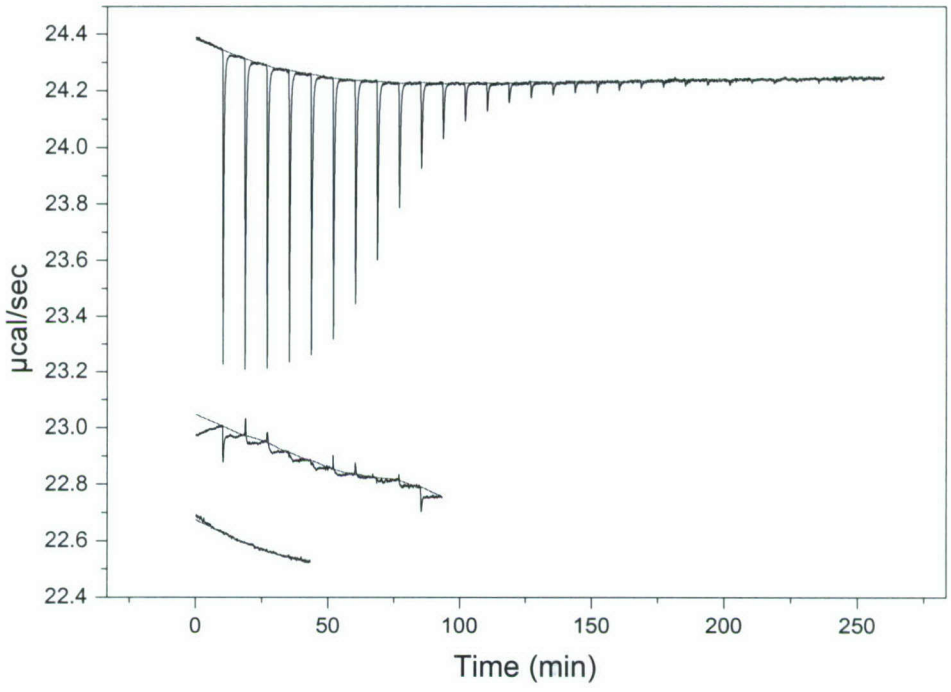

Figure 7. Comparison of the ITC titration profiles for MMTA binding to TmAdoMetDC-WT* (top), TmAdoMetDC-WT (middle), and TmAdoMetDC-S63A (bottom). Profiles were offset along the Y-axis to facilitate comparison.

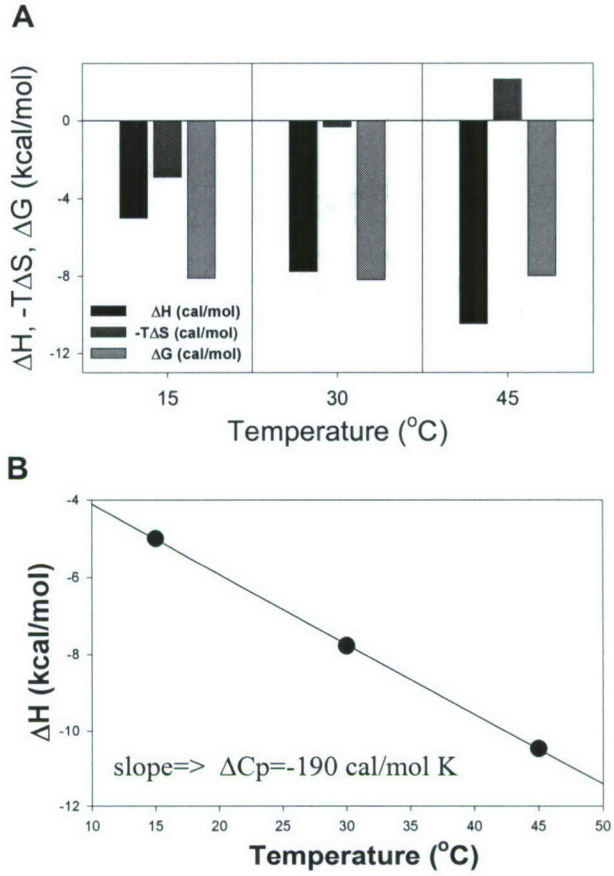

Figure 8. Energetics of MMTA binding to TmAdoMetDC obtained from the ITC experiments. **A.** Comparison of contribution of enthalpy and entropy to the Gibbs energy of binding at different temperature. **B.** Temperature dependence of the enthalpy of binding. The slope of the line in the heat capacity change upon unfolding, ΔC_p, and is estimated to be -190 cal/(mol K). Considering the overall dimension of the ligand (MMTA) such small ΔC_p compared reasonably with the ΔC_p in other reactions involving small ligand such as S100P-melittin, -550 cal/(mol K), or calmodulin-peptide interactions, -860 cal/(mol K).

5. Personnel Supported

Marimar Lopez, PhD.

6. Publications

None (1 in preparation)

7. Interactions/Transitions

a. None

b. None

c. None

8. New Discoveries, Inventions, Patent Disclosures

None

9. Honors/Awards

The 2007 Trustees Celebration of Faculty Achievement Award